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HISTAMINE AND 5-HYDROXYTRYPTAMINE IN THE INTESTINAL TRACT OF GERM-FREE ANIMALS, ANIMALS HARBOURING ONE MICROBIAL SPECIES AND CONVENTIONAL ANIMALS

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Histamine and 5-hydroxytryptamine were determined in the intestinal tract of germ-free and conventional rats and mice. Comparable histamine data were collected in *Clostridium perfringens* mono-associated rats, while 5-hydroxytryptamine determinations were extended to include the chicken. In rats and mice harbouring an intestinal microflora, bacterial formation of histamine occurs mainly in the caecum. Compared to values found in germ-free animals, histamine levels in the wall of the small intestine of the conventional animal tend to be higher, though in the rat the data are not consistent. Mono-association (harbouring of one microbial species) of germ-free rats with a histamine-producing strain of *Clostridium perfringens* resulted in high histamine concentrations in the caecal contents, but failed to increase the histamine levels in the wall of the small intestine. 5-Hydroxytryptamine levels in the intestinal wall in the presence of an intestinal flora were generally lower than those in germ-free animals. Modification of the flora by dietary administration of penicillin to mice partly abolished its depressing effect.

Comparison of the intestinal tract of the germ-free animal with that of its conventional counterpart shows a lower relative weight, partially caused by a lower tissue hydration (Gordon & Wostmann, 1960). The absence of a microbial flora strikingly reduces the various reticulo-endothelial elements, notably the plasma cells and the lymphocytes (Gordon & Bruckner-Kardoss, 1961). Apparently the presence of the flora imparts elements of a mild (physiological) inflammation to the gut as an essential part of the mechanism with which the host animal adapts itself to its environment. Results obtained by Gilbert (1959) and by Des Prez, Fallon & Hook (1960) seem to indicate that, depending on the species, histamine and/or 5-hydroxytryptamine are released upon contact with bacterial endotoxins. Histamine formation is accelerated under the influence of *Escherichia coli* endotoxin (Hinshaw, Jordan & Vick, 1961), evidently through adaptive stimulation of histidine decarboxylase (Schayer, 1962). As both amines are known to induce vasodilatation, increase permeability and produce oedema, they could be important factors in regulating the " adaptive " inflammation. Both histamine and 5-hydroxytryptamine are known to be relatively abundant in the intestinal mucosa of many species, the mucosa being the site of the most intimate contact between the microbial flora and the host.

In this paper we present histamine and 5-hydroxytryptamine values in the intestinal tract of animals reared with and without a " normal " microbial flora.

Some data are given regarding the effect of a microbial population consisting of a single bacterial species on histamine levels in the intestinal tract of the rat.

METHODS

Germ-free rats were obtained from the Lobund Wistar strain germ-free colony. The animals were males of approximately 100 days of age, weighing from 280 to 370 g. Rats mono-associated with *Clostridium perfringens,* type E, were originally taken from the germ-free rat colony and inoculated orally with a viable culture at the age of 29 days. At the time of sacrifice (91 days) the bacterial population of the caecum reached log 9 to log 10 cells per gram of dry caecal content. Genetically closely related controls were taken from the conventional rat colony. All rats received steam sterilized diet L-356 (Larner & Gillespie, 1957), a semi-synthetic diet based on Labco casein 20%, rice flour 58%, corn oil 5%, cellophane spangles 5%, with vitamins, minerals, yeast extract and liver powder added and containing histamine approximately 0.3 μg/g. Water and diet for all animals were available *ad libitum.*

Germ-free mice were obtained from colonies of the Notre Dame 1 strain (Swiss-Webster Harlan origin) and the Notre Dame 2 strain (Swiss-Webster Fort Detrick origin). Genetically closely related controls were taken from conventional colonies. The animals were 80 to 90 days of age, weighing approximately 30 g. All ND-1 strain animals were males; the ND-2 strain mice were of mixed sexes. The ND-1 strain animals received steam sterilized practical type diet L-462 (Wostmann, 1959), based on whole wheat flour 33%, corn meal (yellow) 33%, lactalbumin 10%, casein 5%, whole milk powder 10% with vitamins, minerals, alfalfa meal and liver powder added and containing histamine 1.8 μg/g of diet. ND-2 mice were fed commercial formula 5010C (Ralston Purina Co., St. Louis, Missouri), containing histamine 15 μg/g.

Germ-free White Leghorn chickens were obtained by methods essentially similar to those described by Reyniers, Trexler, Ervin, Wagner, Luckey & Gordon (1949). Control animals derived from eggs from the same clutch were kept in the conventional animal house. At 35 days of age these animals (mixed sexes) weighed approximately 350 g. Both experimental groups were fed steam sterilized diet L-289F, consisting of yellow corn 50%, soybean meal 23%, wheat middlings 9%, caesin 7%, alfalfa 2%, fish meal 2%, meat scraps 2%, minerals 4%, vitamins and carriers 1%.

Rats and mice were killed by cardiac exsanguination under pentobarbitone anaesthesia. Chickens were exsanguinated after electro-shock. Germ-free animals were taken out of the germ-free environment and sacrificed within 1 hr. Tissues were collected in ice-cold acid saline (0.1 N hydrochloric acid) and stored at $-25°$ C. Homogenization was in acid saline. Aliquots of the total homogenates were used in the assays.

Histamine was determined fluorometrically as a condensation product with o-phthalaldehyde after extraction of the alkalinized aliquot (*p*H 12) with normal butanol, addition of heptane to the histamine containing butanol phase and re-extraction into acid (Shore, Burkhalter & Cohn, 1959). In one older series, specifically indicated, histamine was determined using the method of Lowry, Graham, Harris, Priebat, Marks & Bregman (1954). In the case of whole blood (heparinized or oxalated), 0.9 ml. of water was added for each ml. of blood. Subsequently 0.1 ml. 70% perchloric acid was added, the mixture agitated for 10 min and then centrifuged for 20 min. Aliquots of the supernatant were used in the assay.

5-Hydroxytryptamine was determined using the colour reaction with 1-nitroso-2-naphthol and nitrous acid (Udenfriend, Weissbach & Brodie, 1958), modified to our needs. Purification was achieved by extraction of 5-hydroxytryptamine from the alkaline tissue extract with normal butanol (*p*H 10), addition of heptane to the butanol phase and re-extraction of the 5-hydroxytryptamine into acid. All determinations were run within one week after harvesting the material since it was found that, even at $-25°$ C, 5-hydroxytryptamine was lost upon prolonged storage.

All results of both histamine and 5-hydroxytryptamine determinations are expressed in terms of the free base.

RESULTS

Histamine in the wall of the gastro-intestinal tract

Rat. The data in Table 1 are given to demonstrate the variability of the histamine levels in the intestinal wall of the conventional rat. The data in series A–D, gathered over a period of approximately two years, show a pronounced change in histamine concentration which presumably reflects an alteration in the intestinal microflora of the rats housed in the open animal house. During the same time-span,

TABLE 1

HISTAMINE CONTENT OF THE WALL OF THE UPPER HALF OF THE SMALL INTESTINE AND OF THE CAECUM OF GERM-FREE AND CONVENTIONAL MALE RATS (μG/G TISSUE)

Age: 100 days. Diet: L-356. Number of animals in parenthesis. S.D.M. values given. * Series I, II and III and series B, C and D approximately parallel in time. † Determined according to Lowry *et al.* (1954)

Germ-free				Conventional			
Series*	Small int. upper half	Caecum	Ratio	Series*	Small int. upper half	Caecum	Ratio
				A† (10)	36·8 ±3·6	24·7 ±2·2	1·49
I (8)	27·0 ±1·5	24·4 ±1·1	1·11	B (8)	39·6 ±2·7	27·6 ±2·0	1·43
II (5)	27·6 ±2·5	27·7 ±2·0	1·00	C (6)	18·2 ±1·3	13·0 ±2·8	1·40
III (6)	26·4 ±3·0	26·0 ±1·1	1·01	D (8)	23·9 ±2·2	16·7 ±2·2	1·43

the levels found in the germ-free rat (Series I to III) show a remarkable stability. However, the ratios between the concentrations in the wall of those parts of the tract which in the conventional animal harbour the lowest and the highest numbers of viable bacteria, viz., the upper half of the small intestine and the caecum, are remarkably constant within each experimental group.

More detailed results, including the analysis of gastro-intestinal contents, are given in Table 2. The results from germ-free rats, series I, II and III, have been combined for further consideration ; so have series C and D from conventional rats. Histamine concentrations in the wall of the small intestine of the conventional rat were higher than found in germ-free animals in series B (about 43 compared with 21 μg/g) but similar for series C and D (both about 22 μg/g). Concentrations of histamine in the wall of the caecum were lower than those of the small intestine in conventional animals but slightly higher in germ-free animals.

Mouse. The data for the mouse confirm the well-known fact that histamine levels in the gastro-intestinal tract are much lower than those found in the rat. In the case of the strains compared in Table 2 the ratio was approximately 1 to 10. In the conventional mouse the intestinal wall always contained significantly more histamine than found in the germ-free animal (3.9 μg/g as compared with 2.6). The contents of the caecal wall were similar (1.4 μg/g). Unlike the rat, the different series of mouse experiments showed no apparent trend with time.

TABLE 2

HISTAMINE IN THE GASTRO-INTESTINAL TRACT OF GERM-FREE, MONO-ASSOCIATED AND CONVENTIONAL RATS AND GERM-FREE AND CONVENTIONAL MICE

Number of animals in parenthesis. S.D.M. values given. * Including pooled samples

Rat (sex: ♂; age: 100 d.; diet: L-356)	Stomach			Small intestine first half			Small intestine third quarter			Small intestine fourth quarter			Caecum		
	Wall	Contents		Wall	Contents		Wall	Contents		Wall	Contents		Wall	Contents	
	μg/g	μg/g	% dry	μg/g	μg/g	% dry	μg/g	μg/g	% dry	μg/g	μg/g	% dry	μg/g	μg/g	% dry
Germ-free series I+II+III	—	6·0 (6) ±0·5	33·6 ±3·2	27·0 (18) ±1·2	2·0 (5)* ±0·2	18·9 ±0·8	23·6 (17) ±1·3	1·3 (12)* ±0·1	26·2 ±0·9	20·6 (19) ±1·1	1·1 (16) ±0·1	27·0 ±1·0	25·6 (18) ±0·8	1·5 (16) ±0·1	14·0 ±1·0
Conv. series B	—	—	—	39·6 (8) ±2·7	6·5 (7) ±0·7	16·4 ±0·8	46·8 (8) ±2·3	5·3 (7) ±0·4	22·6 ±1·0	44·9 (8) ±3·3	4·8 (7) ±1·0	25·7 ±0·9	27·4 (8) ±2·0	4·6 (7) ±0·7	24·3 ±1·2
Conv. series C+D	—	12·3 (11) ±1·3	32·1 ±1·8	21·5 (14) ±1·5	3·7 (5)* ±0·7	20·5 ±1·1	22·1 (14) ±1·5	3·1 (5)* ±0·4	25·2 ±2·1	22·9 (14) ±1·6	1·3 (9) ±0·1	28·5 ±1·5	15·3 (11) ±2·0	4·7 (13) ±0·7	27·0 ±2·3
Mono-assoc. *Cl. perf.* E	—	—	—	—	—	—	—	—	—	21·2 (13) ±1·3	—	—	—	11·4 (13) ±1·7	16·7 ±0·7

Mouse ND-1 (sex: ♂; age: 80 d.; diet: L-462)	Small intestine	Caecum		
	Wall	Wall	Contents	
	μg/g	μg/g	μg/g	% dry
Germ-free	2·6 (25) ±0·2	1·4 (11)* ±0·2	1·0 (15) ±0·1	15·9 ±0·6
Conv.	3·9 (18) ±0·3	1·4 (4)* ±0·1	3·4 (10)* ±0·3	26·6 ±0·6

Histamine in the contents of the gastro-intestinal tract

The histamine concentration found in the stomach contents was unexpectedly high, with conventional rats showing about twice the amount found in the germ-free animals. In view of the low histamine content of the L-356 diet (0.3 μg/g), this implies that a considerable amount of histamine has been added to (or has been formed in) the food mass, even in the germ-free animal. This amount must be greater in the conventional than in the germ-free group, as the difference between the two seems too great to explain solely on the basis of possible coprophagia. Upon entering the small intestine there is a decrease in dry matter indicating a dilution of the food mass. The data suggest that absorption of histamine takes place at this stage. In the second half of the small intestine further absorption seems to occur, but the comparatively high values found in series B in the contents of the last quarter seem to indicate the possibility of a substantial microbial formation of histamine in the lower ileum. As a contrast, the conventional groups C and D show only the low " background " value also found in the germ-free animal. All data indicate the caecum as the main site of the bacterial formation of histamine, while the results of a limited number of analyses of faecal material (not included in Table 2) imply that little or no histamine is absorbed in the large intestine.

All germ-free rats and mice were found to have the enlarged caecum typical of the germ-free rodent (Gordon & Wostmann, 1960). It contained a much more fluid mass than the caecum of the conventional animal (Table 2). Comparison of dry weight data of the intestinal contents of germ-free and conventional rats showed a comparable condition throughout the gastro-intestinal tract with the exception of the caecum. Here a sudden drop in dry weight in the germ-free animal indicates different hydrodynamic conditions. Analysis of the caecal contents of *Clostridium perfringens* mono-associated rats shows that this condition is not changed by the presence of a bacterium which obviously produces large amounts of histamine (Table 2).

Histamine concentrations in the blood were not affected by the presence or absence of a microbial flora. The levels were 15.6 ± 1.5 and 14.5 ± 1.2 μg/100 ml. whole blood respectively for 10 germ-free and 23 conventional animals.

5-Hydroxytryptamine in the wall of the gastro-intestinal tract

The 5-hydroxytryptamine data obtained from conventional animals were quite consistent during this period and did not show the variation shown by the histamine levels in the intestinal tract of the rat. With the colorimetric method at our disposal, no 5-hydroxytryptamine could be detected in intestinal and caecal contents.

Details of the analysis of the intestinal wall of germ-free and conventional rats, mice and chickens are given in Table 3. These data demonstrate that the presence of a " normal " intestinal flora tends to be associated with a lower 5-hydroxytryptamine level in the intestinal wall. In the mouse this effect is more marked than in the rat, though in both cases the reduction is a significant one. Addition of procaine penicillin to the diet of conventional mice gave rise to values half-way between the germ-free and the conventional levels. The reduction of 5-hydroxy-

TABLE 3

5-HYDROXYTRYPTAMINE IN THE INTESTINAL WALL OF GERM-FREE AND CONVENTIONAL ANIMALS

Number of animals in parenthesis. S.D.M. values given. hs, Significant at 1% level; s, significant at 5% level; s?, significant at 10% level from comparable conventional value or as indicated. * Including pooled samples. † Addition of procaine-penicillin 300 mg/kg of diet. ‡ Product of Ralston Purina

				Germ-free		Conventional	
Species	Age	Diet	Sample small int.	Small int. μg/g	Caecum μg/g	Small int. μg/g	Caecum μg/g
Rat ♂	100 d.	L-356	Upper half	7·7 (18) hs ±0·2		5·5 (18) ±0·2	
			Lower half	6·7 (18) hs ±0·2		5·5 (18) ±0·3	
			Total	7·2 (18) hs ±0·2	5·5 (17) ±0·5	5·5 (18) ±0·2	6·4 (13) ±0·5
Mouse ♂ ND-1	80 d.	L-462	Total	9·1 (11) hs ±0·7	5·0 (12) hs ±0·3	5·0 (22) ±0·2	3·7 (9)* ±0·1
	80 d.	L-462 +PP†	Total	—	—	7·0 (6) ±0·5	
Mouse ♂ ND-2	80 d.	5010C‡	Total	18·8 (7) hs ±1·0	8·4 (6)* hs ±0·7	9·9 (12) ±0·4	4·3 (8) ±0·3
Chicken ♂♀	35 d.	L-289F	Last 8 in.	15·2 (16) s? ±0·5	—	13·7 (20) ±0·6	—

tryptamine levels caused by the microflora presently existing in our conventional chickens is small and its significance questionable.

In the rat, where the presence of an intestinal microflora was associated with a lower 5-hydroxytryptamine concentration in the intestinal wall, the level in the wall of the caecum was apparently unaffected. In the mouse the 5-hydroxytryptamine concentration in both the intestine and caecum was significantly lower.

Where more detailed analysis was possible, as in the rat, the data show that the germ-free animal has a higher concentration of 5-hydroxytryptamine in the upper than in the lower half of the small intestine. A similar trend is demonstrable in the histamine data (Table 2). In both instances the phenomenon is lost in the presence of a microbial flora.

DISCUSSION

In the older literature the opinion prevailed that most histamine found in the body originated from bacterial decarboxylation of histidine (Waton, 1956); lately the emphasis has been on tissue decarboxylation (Schayer, 1960; Telford & West, 1961), its possible control of the adrenal (Telford & West, 1961) and the effect of bacterial toxins on this conversion system (Schayer, 1962). In the case of 5-hydroxytryptamine, only the second mechanism seems of importance. In the germ-free rat, where interfering flora effects are excluded, both histamine and 5-hydroxytryptamine levels in the wall of the small intestine decrease going from the higher to the lower parts. A possible relationship between the production mechanisms of these two amines should therefore be considered.

Histamine values in the contents of the upper small intestine, where little bacterial activity takes place, were found to be much higher than compatible with the concentrations in the diet. This led to the determination of histamine in the stomach contents of a limited number of germ-free and conventional rats. These levels were even higher than those observed in the duodenum, especially in the case of conventional rats. No good explanation can be given for these relatively high values and for the difference between the experimental groups. The histamine concentrations of the mass entering the small intestine were not reflected by the levels found in the intestinal wall.

In conventional rats, bacterial decarboxylation of histidine takes place mainly in the caecum and lower ileum. Again this histamine production does not directly affect the histamine levels found in the intestinal and caecal walls. Series B (conventional rats) shows histamine levels that are higher than in germ-free animals throughout the intestinal wall (but not the caecum). Conventional series C and D, however, display values comparable in those seen in the germ-free series, while the same holds true for the intestinal wall of animals mono-associated with the histamine-producing strain of *Clostridium perfringens*, type E. In the latter series the animals demonstrated to a certain extent the " physiological inflammation " seen in the conventional groups, as measured by the concentration of reticulo-endothelial elements and the water content in the lower ileum. In both the rat and the mouse the data rather suggest that the presence of an intestinal flora causes an increase in histidine-decarboxylase activity, leading to potentially higher histamine concentrations in the wall. However, only the conventional mouse shows consistently higher values than its germ-free counterpart, indicating that in the rat compensatory mechanisms may be activated.

Recent work has indeed indicated that bacterial toxins stimulate the histidine decarboxylation system (Schayer, 1962). Other studies (Gilbert, 1959 ; Des Prez *et al.*, 1960) lead to the speculation that they might enhance the decarboxylation of 5-hydroxytryptophan as well. We therefore anticipated low 5-hydroxytryptamine levels in the intestine of the germ-free animal, speculating that a low 5-hydroxytryptamine concentration might be one of the causes of the characteristic appearance of the germ-free intestinal wall. The data in Table 3 prove, however, that the absence of an intestinal microflora generally gives rise to higher concentrations of 5-hydroxytryptamine in the intestinal wall.

It is of interest to note the marked difference in 5-hydroxytryptamine content of the intestinal wall between ND-1 and ND-2, both mice of Swiss-Webster origin. Though the ND-1 strain was kept on a practical-type diet made in the laboratory, while the ND-2 strain was fed a commercial-type diet, the magnitude of the phenomenon seems to indicate a strain difference rather than an effect of the diet on the 5-hydroxytryptamine level (Sullivan, 1960).

Assuming that bacterial toxins give rise to an accelerated formation of histamine in the gut wall, it could be speculated that an increase in activity of the amine oxidases, triggered by certain elements of a " normal " microbial flora (and their products, possibly including histamine), could counteract this histamine production while at the same time lowering the 5-hydroxytryptamine concentration. This would

enable an interpretation of the "germfree-like" histamine concentrations found in the intestinal wall of the conventional rats in series C and D and also explain the fact that Gustafsson, Kahlson & Rosengren (1957) found essentially no difference in histamine values between germ-free and conventional rats. In this respect, the relatively low histamine values found in the wall of the caecum of the conventional animal, an organ where intense bacterial activity takes place, might be of significance (Tables 1 and 2). The feeding of antibiotics, which is known to give the intestinal tract a more "germfree-like" character, especially in the chicken (Gordon, Wagner & Wostmann, 1957–8; Wostmann, Wagner & Gordon, 1959–60), also increases 5-hydroxytryptamine levels (Table 3; also Sullivan, 1961). As antibiotics suppress certain flora elements (Wagner & Wostmann, 1958–9), conceivably the same organisms responsible for the flora stimulation of the intestinal tract could also cause an increase in amine oxidase activity.

These studies again demonstrate the variability of the effect of a "normal" microflora. This is evidenced not only by the variation with time in the histamine levels in the intestinal tract of the conventional rat but also by the fact that, in our hands, the depressive effect of the intestinal flora on the 5-hydroxytryptamine concentration in the intestinal wall of the chicken was small and of dubious significance (P value 0.10; Table 3). Phillips, Newcomb, Smith & Lachapelle (1961) report considerable depression of 5-hydroxytryptamine levels in the ileum of conventional White Leghorn chickens of approximately the same age as the animals used in this study. Comparison of the data shows that, while there is little difference in the values found in the germ-free chickens in both laboratories, the actual dissimilarity is in the 5-hydroxytryptamine concentrations in the ileum of the animals with a "normal" microbial flora.

The specific differences between the germ-free and the conventional intestinal tract, as they can be described in terms of circulation, hydration and development of reticulo-endothelial system (see introduction), were not paralleled by differences in histamine or 5-hydroxytryptamine concentration as anticipated. It should be pointed out, however, that as the animals in these studies were all in ecological equilibrium, this observation does not exclude these amines from playing a possibly important role in the mobilization of the host's adaptative mechanisms in case of a disturbance of this equilibrium.

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